CREATINE AMIDINOHYDROLASE, PRODUCTION THEREOF AND USE THEREOF

FIELD OF THE INVENTION

The present invention relates to a novel creatine amidinohydrolase, specifically, a novel creatine amidinohydrolase having a very low Km value for creatine, and a method for producing said enzyme. The present invention also relates to a method for the determination of creatine or creatinine in a sample by the use of said enzyme, and a reagent therefor.

BACKGROUND OF THE INVENTION

A creatine and a creatinine are found in blood and urine. A quick and accurate determination of their amounts is very important in making diagnosis of the diseases such as uremia, chronic nephritis, acute nephritis, giantism, tonic muscular dystrophy and the like. For making diagnosis of these diseases, creatine and creatinine in blood, as well as urine are frequently determined quantitatively.

A creatine can be determined by allowing creatine amidinohydrolase and sarcosine oxidase to react on creatine in a sample and determining the amount of the generated hydrogen peroxide by a method for measuring hydrogen peroxide. A creatinine can be determined by allowing creatinine amidohydrolase, creatine amidinohydrolase and sarcosine oxidase to react on creatinine in a sample and determining the generated hydrogen peroxide by a method for measuring hydrogen peroxide.

The creatinine amidohydrolase, creatine amidinohydrolase and sarcosine oxidase are widely found in the world of microorganisms, have been industrially produced and used as reagents for clinical tests.

Yet, the creatine amidinohydrolase produced from various known cell lines show lower heat stability and greater Km value for creatine. For example, an enzyme derived from the bacteria belonging to the genus Bacillus (U.S. Pat. No. 4,420,562) is thermally stable only at a temperature not more than 40° C. An enzyme derived from *Pseudomonas putida* has a smaller apparent Km value for creatine of 1.33 mM [Archives Biochemistry and Biophysics 177, 508–515 (1976)], though the method for determining the activity is different and the Km value for creatine determined by a coupling assay using sarcosine oxidase and peroxidase widely used as reagents for clinical tests, has been unknown. The enzymes derived from the bacteria belonging to the genus Corynebacterium, Micrococcus, Actinobacillus or Bacillus (Japanese Patent Examined Publication No. 76915/1991) is thermally stable at a temperature not more than 50° C., whereas Km value for creatine is as great as about 20 mM, and these enzymes are not suitable for use as reagents for clinical tests.

In an attempt to resolve such problems, the present inventors previously found that the bacteria belonging to the genus Alcaligenes produced a creatine amidinohydrolase which was superior in heat stability and had a relatively smaller Km value (Km value: ca. 15.2) for creatine (Japanese Patent Unexamined Publication No. 63363/1994). Furthermore, they have established a technique for isolating a creatine amidinohydrolase gene having a relatively small Km value for creatine from said bacterial cell line and producing said enzyme in a large amount using Gram negative bacteria as a host (Japanese Patent Application No. 117283/1995).

Moreover, a creatine amidinohydrolase stable in a high pH range and having a small Km value has been reported to be derived from the same genus Alcaligenes cell line (U.S. Pat. No. 5,451,520).

Yet, these creatine amidinohydrolases still have greater Km values as enzymes to be used as routine reagents for clinical tests, and a creatine amidinohydrolase having smaller Km value has been desired.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to produce a novel creatine amidinohydrolase having a small Km value for creatine to the extent sufficient for use as a general reagent for clinical tests, preferably not more than about 15.0, and provide a means for determining creatine or creatinine in a sample using said enzyme.

The present invention is based on the successful provision of a creatine amidinohydrolase gene which expresses a novel creatine amidinohydrolase having a small Km value for creatine, by introducing a mutation, by genetic engineering and protein engineering, into a creatine amidinohydrolase gene derived from conventionally known bacteria belonging to the genus Alcaligenes, which is a known creatine amidinohydrolase having a rather small Km value. The creatine amidinohydrolase of the present invention can be produced in large amounts by culturing a microorganism capable of expressing said gene in a nutrient medium.

The novel creatine amidinohydrolase of the present invention has a very small Km value for creatine as compared to conventionally known enzymes, and shows superior reactivity to creatine contained in a trace amount in a sample. Thus, it is useful as a reagent for determining creatine or creatinine with high sensitivity and high precision.

Accordingly, the present invention provides a novel creatine amidinohydrolase having the following physicochemical properties.

Action: catalyzing the following reaction:
creatine+H_2O→sarcosine+urea

Optimum temperature: ca. 40–50° C.

Optimum pH: ca. 8.0–9.0

Heat stability: stable at not more than about 50° C. (pH 7.5, 30 min)

Km value relative to creatine in a coupling assay using a sarcosine oxidase and a peroxidase: ca. 3.5–10.0 mM

Molecular weight: ca. 43,000 (SDS-PAGE)

Isoelectric point: ca. 3.5

The present invention also provides a method for producing said creatine amidinohydrolase, comprising culturing a microorganism capable of producing a novel creatine amidinohydrolase having the following physicochemical properties, in a nutrient medium, and harvesting said creatine amidinohydrolase from the culture.

Action: catalyzing the following reaction:
creatine+H_2O→sarcosine+urea

Optimum temperature: ca. 40–50° C.

Optimum pH: ca. 8.0–9.0

Heat stability: stable at not more than about 50° C. (pH 7.5, 30 min)

Km value relative to creatine in a coupling assay using a sarcosine oxidase and a peroxidase: ca. 3.5–10.0 mM

Molecular weight: ca. 43,000 (SDS-PAGE)

Isoelectric point: ca. 3.5

The present invention further provides a reagent for determining creatine in a sample, comprising the above-said creatine amidinohydrolase, sarcosine oxidase and a compo-

sition for detection of hydrogen peroxide, and a method for determining creatine in a sample by the use of said reagent.

The present invention further provides a reagent for determining creatinine in a sample, comprising a creatinine amidohydrolase, the above-mentioned creatine amidinohydrolase, sarcosine oxidase and a composition for detection of hydrogen peroxide, and a method for determining creatinine in a sample by the use of said reagent.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a physical map of recombinant plasmid pCRH273.

FIG. 2 shows the time course determination results of creatinine in a sample, by the use of the creatine amidinohydrolase of the present invention and a wild creatine amidinohydrolase.

DETAILED DESCRIPTION OF THE INVENTION

One embodiment of the present invention is a novel creatine amidinohydrolase having the following physicochemical properties.

Action: catalyzing the following reaction:
creatine+H_2O→sarcosine+urea

Optimum temperature: ca. 40–50° C.

Optimum pH: ca. 8.0–9.0

Heat stability: stable at not more than about 50° C. (pH 7.5, 30 min)

Km value relative to creatine in a coupling assay using a sarcosine oxidase and a peroxidase: ca. 4.5±1.0 mM

Molecular weight: ca. 43,000 (SDS-PAGE)

Isoelectric point: ca. 3.5

Another embodiment of the present invention is a novel creatine amidinohydrolase having the following physicochemical properties.

Action: catalyzing the following reaction:
creatine+H_2O→sarcosine+urea

Optimum temperature: ca. 40–50° C.

Optimum pH: ca. 8.0–9.0

Heat stability: stable at not more than about 50° C. (pH 7.5, 30 min)

Km value relative to creatine in a coupling assay using a sarcosine oxidase and a peroxidase: ca. 6.5±1.0 mM

Molecular weight: ca. 43,000 (SDS-PAGE)

Isoelectric point: ca. 3.5

A still another embodiment of the present invention is a novel creatine amidinohydrolase having the following physicochemical properties.

Action: catalyzing the following reaction:
creatine+H_2O→sarcosine+urea

Optimum temperature: ca. 40–50° C.

Optimum pH: ca. 8.0–9.0

Heat stability: stable at not more than about 50° C. (pH 7.5, 30 min)

Km value relative to creatine in a coupling assay using a sarcosine oxidase and a peroxidase: ca. 9.0±1.0 mM

Molecular weight: ca. 43,000 (SDS-PAGE)

Isoelectric point: ca. 3.5

One method for producing the creatine amidinohydrolase of the present invention comprises mutation of a gene encoding a wild creatine amidinohydrolase by genetic engineering and protein engineering method, generating a mutant DNA encoding a novel creatine amidinohydrolase having a smaller Km value for creatine than the wild creatine amidinohydrolase, expressing said DNA in a suitable host and harvesting the creatine amidinohydrolase thus produced.

While the gene encoding a wild creatine amidinohydrolase which is to be mutated is not particularly limited, in one embodiment of the present invention, it is the creatine amidinohydrolase gene depicted in the Sequence Listing•SEQ ID:No.2, which is derived from *Alcaligenes•faecalis* TE3581 (FERM P-14237).

In another embodiment of the present invention, a novel creatine amidinohydrolase having a smaller Km value for creatine than a wild creatine amidinohydrolase is produced by mutating the gene encoding the amino acid sequence depicted in the Sequence Listing•SEQ ID:No.1.

A wild creatine amidinohydrolase gene can be mutated by any known method. For example, a wild creatine amidinohydrolase DNA or a microorganism cells having said gene is brought into contact with a mutagenic agent, or ultraviolet irradiation is applied, or a protein engineering method is used such as PCR and site-directed mutagenesis. Alternatively, an *Escherichia coli* susceptible to gene mutation at high frequency due to defective gene repair mechanism may be transformed with a wild creatine amidinohydrolase gene DNA for mutation in vivo.

For example, *Escherichia coli* is transformed with the mutant creatine amidinohydrolase gene obtained above and plated on a creatine amidinohydrolase activity detection agar medium [J. Ferment. Bioeng., Vol. 76 No. 2 77–81(1993)], and the colonies showing clear color development are selected. The selected colonies are inoculated to a nutritive medium (e.g., LB medium and 2×YT medium) and cultured overnight at 37° C. The cells are disrupted and a crude enzyme solution is extracted.

The method for disrupting the cells may be any known method, such as physical rupture (e.g., ultrasonication and glass bead rupture), as well as by the use of a lysozyme. This crude enzyme solution is used to determine the creatine amidinohydrolase activity of two kinds of activity determination reaction solutions having different substrate concentrations. Comparison of the activity ratios of the two with that obtained using a wild creatine amidinohydrolase leads to the screening of the creatine amidinohydrolase having smaller Km value.

The method for obtaining the purified creatine amidinohydrolase from the cell line selected as above may be any known method, such as the following.

After the cells obtained by culturing in a nutrient medium are recovered, they are ruptured by an enzymatic or physical method and extracted to give a crude enzyme solution. A creatine amidinohydrolase fraction is recovered from the obtained crude enzyme solution by ammonium sulfate precipitation. The enzyme solution is subjected to desalting by Sephadex G-25 (Pharmacia Biotech) gel filtration and the like.

After this operation, the resulting enzyme solution is separated and purified by octyl Sepharose CL-6B (Pharmacia Biotech) column chromatography to give a standard purified enzyme product. This product is purified to the degree that it shows almost a single band by SDS-PAGE.

The microorganism to be used in the present invention to produce the novel creatine amidinohydrolase is exemplified by *Escherichia coli* JM109 (pCRH273M1) (FERM BP-5374), *Escherichia coli* JM109 (pCRH273M2) (FERM BP-5375), *Escherichia coli* JM109 (pCRH273M3) (FERM BP-5376) and the like.

The method for culturing these microorganisms and recovering the creatine amidinohydrolase of the present

invention from the cultures thereof are not particularly limited, and conventional methods can be applied.

The novel creatine amidinohydrolase obtained by the above-mentioned production method of the present invention has the following physicochemical properties.

Action: catalyzing the following reaction:
creatine+H_2O→sarcosine+urea

Optimum temperature: ca. 40–50° C.

Optimum pH: ca. 8.0–9.0

Heat stability: stable at not more than about 50° C. (pH 7.5, 30 min)

Km value relative to creatine in a coupling assay using a sarcosine oxidase and a peroxidase: ca. 3.5–10.0 mM

Molecular weight: ca. 43,000 (SDS-PAGE)

Isoelectric point: ca. 3.5

The Km value in the present invention is the value relative to creatine in a coupling assay using a sarcosine oxidase and a peroxidase. While the conventional enzyme derived from *Pseudomonas putida* has a small apparent Km value for creatine of 1.33 mM [Archives Biochemistry and Biophysics 177, 508–515 (1976)], the activity is determined by measuring the residual creatine in the reaction mixture with α-naphthol and diacetyl, and the Km value for creatine by a coupling assay using a sarcosine oxidase and a peroxidase, which are widely used as reagents for clinical tests, has been unknown.

The creatine amidinohydrolase of the present invention can be used for the determination of creatine upon combination with a sarcosine oxidase and a composition for detection of hydrogen peroxide. Moreover, when creatinine amidohydrolase is concurrently used, creatinine can be determined as well.

The determination method of the present invention utilizes the following reactions.

Reaction 1:

$$\text{creatine} + H_2O \xrightarrow{\text{creatine amidinohydrolase}} \text{sarcosine} + \text{urea}$$

Reaction 2:

$$\text{sarcosine} + O_2 + H_2O \xrightarrow{\text{sarcosine oxidase}} \text{glycine} + H_2O_2 + \text{formaldehyde}$$

Reaction 3:

$$H_2O_2 + \text{hydrogen receptor} + \text{coupler} \xrightarrow{\text{peroxidase}} \text{quinonimine pigment}$$

When creatinine is determined, the following reaction is further utilized.

Reaction 4:

$$\text{creatinine} + H_2O \xrightarrow{\text{creatinine amidohydrolase}} \text{creatine}$$

The quinonimine pigment produced is generally subjected to the determination of absorbance at 500–650 nm wavelength. The method for determining creatine is an end method or a rate method, though the end method is generally used.

The inventive creatine amidinohydrolase having smaller Km value can reduce the amount of the enzyme to be used in the test reagent for creatine or creatinine determination to about ⅓–¼ as compared to the necessary amount of conventional enzymes, and achieves good reactivity in the latter half of the reaction.

The reagent for determining creatine in a sample of the present invention contains the above-mentioned creatine amidinohydrolase, sarcosine oxidase, and a composition for detecting hydrogen peroxide.

The reagent for determining creatinine in a sample of the present invention contains a creatinine amidohydrolase, the above-mentioned creatinine amidohydrolase, sarcosine oxidase, and a composition for detecting hydrogen peroxide.

The sarcosine oxidase to be used for detecting creatine or creatinine of the present invention can be obtained from the microorganisms originated from the genera Arthrobacter, Corynebacterium, Alcaligenes, Pseudomonas, Micrococcus, Bacillus and the like, and some of them are commercially available.

The creatinine amidohydrolase can be obtained from the microorganisms originated from the genera Pseudomonas, Flavobacterium, Alcaligenes, Penicillium and the like, and some of them are commercially available.

The composition for the detection of hydrogen peroxide contains an enzyme having a peroxidase activity, chromophore and a buffer. The enzyme having a peroxidase activity is exemplified by peroxidase, haloperoxidase, bromoperoxidase, lactoperoxidase, myeloperoxidase and the like. The chromophore comprises a hydrogen receptor and a coupler. The hydrogen receptor may be any as long as it receives hydrogen in the reaction with hydrogen peroxide, peroxidase and a coupler, which is specifically exemplified by 4-aminoantipyrine, 3-methyl-2-benzothiazolinehydrazine derivative and the like. Examples of the coupler include aniline derivatives such as aniline and N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine (TOOS), phenol derivatives such as phenol and p-chlorophenol, and the like.

The reagent for the determination of creatine of the present invention contains each ingredient in a preferable proportion of creatine amidinohydrolase ca. 5–300 U/ml, sarcosine oxidase ca. 1–100 U/ml, peroxidase ca. 0.01–50 U/ml, hydrogen donor ca. 0.1–10 mM, and a coupler ca. 0.1–50 mM.

The reagent for the determination of creatinine of the present invention contains each ingredient in a preferable proportion of creatinine amidohydrolase ca. 10–300 U/ml, creatine amidinohydrolase ca. 10–300 U/ml, sarcosine oxidase ca. 1–100 U/ml, peroxidase ca. 0.01–50 U/ml, hydrogen donor ca. 0.1–10 mM, and a coupler ca. 0.1–50 mM.

The reagent for the determination of creatine or creatinine of the present invention is generally used with a buffer having a pH of about 6–8. Examples of the buffer include phosphate buffer, Good buffer, Tris buffer and the like.

Where necessary, ascorbate oxidase or catalase may be added to the reagent of the present invention. Other compounds may be also added to the reagent of the present invention for smooth enzyme reaction and color development. Such compounds are, for example, stabilizers, surfactants, excipients and the like.

EXAMPLES

The present invention is described in detail by way of the following Examples.

In the Examples, the activity of creatine amidinohydrolase was determined as follows. The enzyme activity in the present invention is defined to be the enzyme amount capable of producing 1 μmole of sarcosine per min under the following conditions being one unit (U).

Reaction mixture composition	
0.3 H	HEPES pH 7.6
0.005%	4-aminoantipyrine
0.015%	phenol
1.8%	creatine
6 U/ml	sarcosine oxidase
6 U/ml	peroxidase

The above-mentioned reaction mixture (3 ml) is taken with a cuvette (d=1 cm) and preliminarily heated to 37° C. for about 3 minutes. An enzyme solution (0.1 ml) is added, and the mixture is gently admixed. Using water as a control, changes in absorbance at 500 nm are recorded for 5 minutes using a spectrophotometer controlled to 37° C. Based on the linear portion of 2–5 minutes thereof, changes in absorbance per minute are determined (ΔOD test).

The blank test is performed in the same manner as above except that a solution (0.1 ml, 50 mM potassium phosphate buffer, pH 7.5) for diluting the enzyme is used instead of the enzyme solution and changes in absorbance per minute are determined (ΔOD blank).

The enzyme amount is calculated by inserting each measure into the following formula.

$$U/\text{ml} = \frac{\Delta OD/\text{min}\ (\Delta OD\ \text{test} - \Delta OD\ \text{blank}) \times 3.1 \times \text{dilution fold}}{13.3 \times 1/2 \times 1.0 \times 0.1}$$

wherein each constant denotes the following:

13.3: millimolar absorbance coefficient ($cm^2/\mu M$) under the above measurement conditions of quinonimine pigment

½: coefficient indicating that the quinonimine pigment formed from one molecule of hydrogen peroxide generated in the enzyme reaction is ½ molecule

1.0: light path length (cm)

0.1: amount of enzyme added (ml)

Reference Example 1

Isolation of chromosomal DNA

The chromosomal DNA of *Alcaligenes•faecalis* TE3581 was isolated by the following method.

The cells (FERM P-14237) were shake-cultured overnight at 30° C. in a nutrient broth (150 ml) and the cells were collected by centrifugation (8000 rpm, 10 min). The cells were suspended in a solution (5 ml) containing 10% sucrose, 50 mM Tris-HCl (pH 8.0) and 50 mM EDTA, and a lysozyme solution (1 ml, 10 mg/ml) was added. The mixture was incubated at 37° C. for 15 min. Then, 10% SDS solution (1 ml) was added. An equivalent amount (1 ml) of a chloroform•phenol solution (1:1) was added to this mixture. The mixture was stirred and separated into an aqueous layer and a solvent layer by centrifugation at 10,000 rpm for 3 min. The aqueous layer was separated, and onto this aqueous layer was gently layered a 2-fold amount of ethanol. The content was slowly stirred with a glass rod to allow the DNA to wind around the rod.

This DNA was dissolved in 10 mM Tris-HCl solution (pH 8.0, hereinafter abbreviated as TE) containing 1 mM EDTA. This solution was treated with an equivalent amount of chloroform•phenol solution. The aqueous layer was separated by centrifugation, and a 2-fold amount of ethanol was added. The DNA was separated again by the method described above and dissolved in 2 ml of TE.

Reference Example 2

Preparation of DNA fragment containing a gene encoding creatinine amidinohydrolase and recombinant vector containing said DNA fragment

The DNA (20 μg) obtained in Reference Example 1 was partially cleaved with restriction enzyme Sau3AI (Toyo Boseki Kabushiki Kaisha) and 2–10 kbp fragments were recovered by sucrose density gradient centrifugation. Meanwhile, pBluescript KS(+) cleaved with restriction enzyme BamHI (Toyo Boseki Kabushiki Kaisha) was dephosphorylated with bacterial alkaline phosphatase (Toyo Boseki Kabushiki Kaisha). Then, the both DNAs were treated with T4DNA ligase (1 unit, Toyo Boseki Kabushiki Kaisha) at 16° C. for 12 hr to ligate the DNA. *Escherichia coli* JM109 competent cell (Toyo Boseki Kabushiki Kaisha) was transformed with the ligated DNA and plated onto a creatine amidinohydrolase activity detection agar medium [0.5% yeast extract, 0.2% meat extract, 0.5% polypeptone, 0.1% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4/7H_2O$, 1.15% creatine, 10 U/ml sarcosine oxidase (Toyo Boseki Kabushiki Kaisha), 0.5 U/ml peroxidase (Toyo Boseki Kabushiki Kaisha), 0.01% o-dianisidine, 50 μg/ml ampicillin and 1.5% agar]. The activity of creatine amidinohydrolase was detected using, as the indices, the colonies grown in the above-mentioned medium and stained in brown. The colonies (ca. 1×10^5) of the transformant were obtained per DNA 1 μg used.

About 12,000 colonies were screened, and 6 colonies were found stained in brown. These strains were cultured in LB liquid medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, 50 μg/ml ampicillin) and creatine amidinohydrolase activity was determined, as a result of which creatine amidinohydrolase activity was detected in every strain. The plasmid of the strain which showed the highest creatine amidinohydrolase activity contained ca. 5 kbp insert DNA fragment, and this plasmid was named pCRH17.

Then, the insert DNA of pCRH17 was cleaved with restriction enzymes EcoRV (Toyo Boseki Kabushiki Kaisha) and PstI (Toyo Boseki Kabushiki Kaisha), and ligated to pBluescript KS(+) cleaved with said restriction enzymes to prepare pCRH173.

Example 1

Preparation of recombinant plasmid pCRH273 by mutating creatine amidinohydrolase gene

The region of from β-galactosidase structural gene derived from the vector to the upstream region of the creatine amidinohydrolase structural gene of the insert DNA was deleted from the recombinant plasmid pCRH173 of Reference Example 2, using the synthetic DNA depicted in SEQ ID:No.3 and a commercially available mutation introduction kit (Transformer™; Clonetech) to prepare recombinant plasmid pCRH173M. The detailed method for introducing the mutation was given in the protocol attached to the kit.

The pCRH173M was cleaved with restriction enzyme EcoRI (Toyo Boseki Kabushiki Kaisha) and self-ligated to prepare pCRH273 (FIG. 1).

Example 2

Selection of candidate cell lines producing the objective mutant creatine amidinohydrolase

A commercially available *Escherichia coli* competent cell (*E. coli* XL1-Red; Clonetech) was transformed with the pCRH273 prepared in Example 1, and the entire amount thereof was inoculated to 3 ml of LB liquid medium (1% polypeptone, 0.5% yeast extract, 1.0% NaCl) containing ampicillin (50 μg/ml; Nakarai Tesque), which was followed

by shake culture overnight at 37° C. A plasmid was recovered from the entire amount of this culture by a conventional method. The commercially available *Escherichia coli* competent cell (*E. coli* JM109, Toyo Boseki Kabushiki Kaisha) was transformed again with this plasmid and plated onto a creatine amidinohydrolase activity detection agar medium, which was then incubated overnight at 37° C. The cell lines which showed a strong expression of the creatine amidinohydrolase activity, i.e., the strains which showed a deep color development, were selected from the mutant creatine amidinohydrolase library thus obtained.

Example 3

Screening of creatine amidinohydrolase-producing cell line having a reduced Km value

The candidate cell lines selected in Example 2 were inoculated to 3 ml of TB medium (1.2% polypeptone, 2.4% yeast extract, 0.4% glycerol, 0.0231% KH_2PO_4, 0.1254% K_2HPO_4) containing ampicillin (200 μg/ml) and shake-cultured overnight at 37° C. The cells were recovered from 1 ml of the culture by centrifugation, and a crude enzyme solution was prepared therefrom by rupture with glass beads. Using the crude enzyme solution thus obtained and following the above-mentioned activity determination method, creatine amidinohydrolase was determined. Meanwhile, using an activity determination reagent having a 1/10 substrate concentration, the creatine amidinohydrolase activity was determined in the same manner. The cell line wherein the ratio of the two kinds of the activity measures (activity with 1/10 substrate concentration÷activity obtained by conventional manner) increased beyond that of a wild creatine amidinohydrolase was selected as a mutant having a reduced Km value.

About 20,000 cell lines were screened by the above method, and three mutant cell lines having a smaller Km value for creatine were obtained, and the respective recombinant plasmids thereof were named pCRH273M1 (FERM BP-5374), pCRH273M2 (FERM BP-5375) and pCRH273M3 (FERM BP-5376).

Example 4

Preparation of creatine amidinohydrolase from *Escherichia coli* JM109 (pCRH273M1)

TB medium (6 L) was dispensed to 10 L jar fermentors, and subjected to autoclaving at 121° C. for 15 min. After allowing them to cool, 50 mg/ml ampicillin (Nakarai Tesque) and 200 mM IPTG (Nippon Seika Corp.), which had been separately sterilized by filtration, were added by 6 ml each. To this medium was added 60 ml of the culture of *Escherichia coli* JM109 (pCRH273M1)(FERM BP-5374) after previous shake culture at 30° C. for 24 hr, which was followed by aeration culture at 37° C. for 24 hr. The activity of creatine amidinohydrolase after the completion of the culture was 8.7 U/ml.

The above-mentioned cells were collected by centrifugation, and suspended in 50 mM phosphate buffer, pH 7.0.

The cells in this suspension were ruptured with a French press and subjected to centrifugation to give a supernatant. The obtained crude enzyme solution was subjected to ammonium sulfate fractionation, desalting with Sephadex G-25 (Pharmacia Biotech) gel filtration and purified by octyl Sepharose CL-6B (Pharmacia Biotech) column chromatography to give a purified enzyme product. The standard creatine amidinohydrolase product obtained by this method showed a nearly single band by SDS-PAGE and had a specific activity then of 18.4 U/mg protein.

Table 1 shows the purification performed so far. Table 2 shows physicochemical properties of the creatine amidinohydrolase obtained by the above methods.

TABLE 1

Purification of creatine amidinohydrolase from *Escherichia coli* JM109 (pCRH273M1)

Step	Total activity (U)	Specific activity (U/mg-protein)	Yield (%)
French press rupture	52200		100.0
$(NH_4)_2SO_4$ precipitation - redissolution	49746	8.3	95.3
Sephadex G-25	46927	10.3	89.9
Octyl Sepharose CL-6B	33094	18 4	63.4

TABLE 2

Physicochemical properties of creatine amidinohydrolase purified from *Escherichia coli* JM109 (pCRH273M1)

Item	Physicochemical properties
Action	creatine + H_2O → sarcosine + urea
Optimal temperature	ca. 40° C.–50° C.
Optimal pH	ca. 8.0–9.0
Thermal stability	ca. 50° C. (50 mM potassium phosphate buffer, pH 7.5, 30 min treatment)
pH stability	ca. 5–8 (40° C., 18 hr preservation)
Km value	ca. 6.5 mM (creatine)
Molecular weight	ca. 43,000 (SDS-PAGE)
Isoelectric point	ca. 3.5 (isoelectric focusing)

Example 5

Preparation of creatine amidinohydrolase from *Escherichia coli* JM109 (pCRH273M2)

TB medium (6 L) was dispensed to 10 L jar fermentors, and subjected to autoclaving at 121° C. for 15 min. After allowing them to cool, 50 mg/ml ampicillin (Nakarai Tesque) and 200 mM IPTG (Nippon Seika Corp.), which had been separately sterilized by filtration, were added by 6 ml each. To this medium was added 60 ml of the culture of *Escherichia coli* JM109 (pCRH273M2)(FERM BP-5375) after previous shake culture at 30° C. for 24 hr, which was followed by aeration culture at 37° C. for 24 hr. The activity of creatine amidinohydrolase after the completion of the culture was 5.6 U/ml.

The above-mentioned cells were collected by centrifugation, and suspended in 50 mM phosphate buffer, pH 7.0.

The cells in this suspension were ruptured with a French press and subjected to centrifugation to give a supernatant. The obtained crude enzyme solution was subjected to ammonium sulfate fractionation, desalting with Sephadex G-25 (Pharmacia Biotech) gel filtration and purified by octyl Sepharose CL-6B (Pharmacia Biotech) column chromatography to give a purified enzyme product. The standard creatine amidinohydrolase product obtained by this method showed a nearly single band by SDS-PAGE and had a specific activity then of 14.3 U/mg protein.

Table 3 shows the purification performed so far. Table 4 shows physicochemical properties of the creatine amidinohydrolase obtained by the above methods.

TABLE 3

Purification of creatine amidinohydrolase from *Escherichia coli* JM109 (pCRH273M2)			
Step	Total activity (U)	Specific activity (U/mg-protein)	Yield (%)
French press rupture	33600		100.0
$(NH_4)_2SO_4$ precipitation - redissolution	25636	7.2	76.3
Sephadex G-25	24326	9.8	72 4
Octyl Sepharose CL-6B	19689	14.3	58.6

TABLE 4

Physicochemical properties of creatine amidinohydrolase purified from *Escherichia coli* JM109 (pCRH273M2)	
Item	Physicochemical properties
Action	creatine + H_2O → sarcosine + urea
Optimal temperature	ca. 45° C.–50° C.
Optimal pH	ca. 8.0–9.0
Thermal stability	ca. 40° C. (50 mM potassium phosphate buffer, pH 7.5, 30 min treatment)
pH stability	ca. 5–8 (40° C., 18 hr preservation)
Km value	ca. 4.5 mM (creatine)
Molecular weight	ca. 43,000 (SDS-PAGE)
Isoelectric point	ca. 3.5 (isoelectric focusing)

Example 6

Preparation of creatine amidinohydrolase from *Escherichia coli* JM109 (pCRH273M3)

TB medium (6 L) was dispensed to 10 L jar fermentors, and subjected to autoclaving at 121° C. for 15 min. After allowing them to cool, 50 mg/ml ampicillin (Nakarai Tesque) and 200 mM IPTG (Nippon Seika Corp.) which had been separately sterilized by filtration were added by 6 ml each. To this medium was added 60 ml of culture of *Escherichia coli* JM109 (pCRH273M3)(FERM BP-5376) after previous shake culture at 30° C. for 24 hr, which was followed by aeration culture at 37° C. for 24 hr. The activity of creatine amidinohydrolase after the completion of the culture was 8.3 U/ml.

The above-mentioned cells were collected by centrifugation, and suspended in 50 mM phosphate buffer, pH 7.0.

The cells in this suspension were ruptured with a French press and subjected to centrifugation to give a supernatant. The obtained crude enzyme solution was subjected to ammonium sulfate fractionation, desalting by Sephadex G-25 (Pharmacia Biotech) gel filtration and purified by octyl Sepharose CL-6B (Pharmacia Biotech) column chromatography to give a purified enzyme product. The standard creatine amidinohydrolase product obtained by this method showed a nearly single band by SDS-PAGE and had a specific activity then of 14.8 U/mg protein.

Table 5 shows the purification performed so far. Table 6 shows physicochemical properties of the creatine amidinohydrolase obtained by the above methods.

TABLE 5

Purification of creatine amidinohydrolase from *Esherichia coli* JM109 (pCRH273M3)			
Step	Total activity (U)	Specific activity (U/mg-protein)	Yield (%)
French press rupture	49800		100.0
$(NH_4)_2SO_4$ precipitation - redissolution	43027	8.3	86.4
Sephadex G-25	39989	9.9	80.3
Octyl Sepharose CL-6B	32021	14.8	64.3

TABLE 6

Physicochemical properties of creatine amidinohydrolase purified from *Escherichia coli* JM109 (pCRH273M3)	
Item	Physicochemical properties
Action	creatine + H_2O → sarcosine + urea
Optimal temperature	ca. 40° C.–45° C.
Optimal pH	ca. 8.0–9.0
Thermal stability	ca. 40° C. (50 mM potassium phosphate buffer, pH 7.5, 30 min treatment)
pH stability	ca. 5–8 (40° C., 18 hr preservation)
Km value	ca. 9.0 mM (creatine)
Molecular weight	ca. 43,000 (SDS-PAGE)
Isoelectric point	ca. 3.5 (isoelectric focusing)

The following Table 7 summarizes the Km values for creatine of the novel creatine amidinohydrolases of the present invention and wild creatine amidinohydrolase. As is evident from Table 7, the novel creatine amidinohydrolases of the present invention had reduced Km values as compared to the wild creatine amidinohydrolase.

TABLE 7

Enzyme	Km value
wild	15.2 mM
pCRH273M1	6.5 mM
pCRH273M2	4.5 mM
pCRH273M3	9 0 mM

Example 7

Using the purified creatine amidinohydrolase prepared in Example 5 and wild creatine amidinohydrolase, a creatinine determination reagent having the following composition was prepared, and the amounts of the creatine amidinohydrolase necessary for giving a creatinine determination reagent was compared.

creatine amidinohydrolase of Example 5 or wild creatine amidinohydrolase	20, 40, 60 U/ml
creatinine amidohydrolase	150 U/ml
sarcosine oxidase	7 U/ml
peroxidase	3 PU/ml
MOPS buffer	0.1 M, pH 8.0
Triton X-100	0.1%
4-aminoantipyrine	0.15 mM
TOOS (aniline derivative)	0.2 mM

The above-mentioned solution (3 ml) was added to a sample (60 μl) containing creatinine (100 mg/dl) and changes in absorbance were determined at 37° C. at wavelength 546 nm. The time course results are shown in FIG. 2.

In the Figure, "Wild" shows a wild creatine amidinohydrolase and "pCRH273M2" is the creatine amidinohydrolase of the present invention.

As is evident from FIG. 2, when the determination was ended in 5 minutes, the creatine amidinohydrolase of the present invention enabled determination with less enzyme amount (ca. ⅓ amount) as compared to the wild creatine amidinohydrolase. It was also confirmed that the reactivity during the latter half of the determination, i.e., when the creatine in the sample decreased, was fine.

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                         SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (iii) NUMBER OF SEQUENCES: 3

(2) INFORMATION FOR SEQ ID NO: 1:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 404 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:
          (A) DESCRIPTION: protein

    (vi) ORIGINAL SOURCE:
          (A) ORGANISM: Alcaligenes faecalis
          (B) STRAIN: TE3581 (FERM P-14237)

    (ix) FEATURE:
          (A) NAME/KEY: mat peptide
          (B) LOCATION: 1 to 404
          (D) OTHER INFORMATION: protein having creatine amidino-
               hydrolase activity

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Met Thr Asp Asp Met Leu His Val Met Lys Trp His Asn Gly Glu Lys
 1               5                  10                  15

Asp Tyr Ser Pro Phe Ser Asp Ala Glu Met Thr Arg Arg Gln Asn Asp
            20                  25                  30

Val Arg Gly Trp Met Ala Lys Asn Asn Val Asp Ala Ala Leu Phe Thr
        35                  40                  45

Ser Tyr His Cys Ile Asn Tyr Tyr Ser Gly Trp Leu Tyr Cys Tyr Phe
    50                  55                  60

Gly Arg Lys Tyr Gly Met Val Ile Asp His Asn Asn Ala Thr Thr Ile
65                  70                  75                  80

Ser Ala Gly Ile Asp Gly Gly Gln Pro Trp Arg Arg Ser Phe Gly Asp
                85                  90                  95

Asn Ile Thr Tyr Thr Asp Trp Arg Arg Asp Asn Phe Tyr Arg Ala Val
            100                 105                 110

Arg Gln Leu Thr Thr Gly Ala Lys Arg Ile Gly Ile Glu Phe Asp His
        115                 120                 125

Val Asn Leu Asp Phe Arg Arg Gln Leu Glu Glu Ala Leu Pro Gly Val
    130                 135                 140

Glu Phe Val Asp Ile Ser Gln Pro Ser Met Trp Met Arg Thr Ile Lys
145                 150                 155                 160

Ser Leu Glu Glu Gln Lys Leu Ile Arg Glu Gly Ala Arg Val Cys Asp
                165                 170                 175

Val Gly Gly Ala Ala Cys Ala Ala Ala Ile Lys Ala Gly Val Pro Glu
            180                 185                 190

His Glu Val Ala Ile Ala Thr Thr Asn Ala Met Ile Arg Glu Ile Ala
        195                 200                 205

Lys Ser Phe Pro Phe Val Glu Leu Met Asp Thr Trp Thr Trp Phe Gln
    210                 215                 220
```

-continued

Ser Gly Ile Asn Thr Asp Gly Ala His Asn Pro Val Thr Asn Arg Ile
225 230 235 240

Val Gln Ser Gly Asp Ile Leu Ser Leu Asn Thr Phe Pro Met Ile Phe
245 250 255

Gly Tyr Tyr Thr Ala Leu Glu Arg Thr Leu Phe Cys Asp His Val Asp
260 265 270

Asp Ala Ser Leu Asp Ile Trp Glu Lys Asn Val Ala Val His Arg Arg
275 280 285

Gly Leu Glu Leu Ile Lys Pro Gly Ala Arg Cys Lys Asp Ile Ala Ile
290 295 300

Glu Leu Asn Glu Met Tyr Arg Glu Trp Asp Leu Leu Lys Tyr Arg Ser
305 310 315 320

Phe Gly Tyr Gly His Ser Phe Gly Val Leu Cys His Tyr Tyr Gly Arg
325 330 335

Glu Ala Gly Val Glu Leu Arg Glu Asp Ile Asp Thr Glu Leu Lys Pro
340 345 350

Gly Met Val Val Ser Met Glu Pro Met Val Met Leu Pro Glu Gly Met
355 360 365

Pro Gly Ala Gly Gly Tyr Arg Glu His Asp Ile Leu Ile Val Gly Glu
370 375 380

Asp Gly Ala Glu Asn Ile Thr Gly Phe Pro Phe Gly Pro Glu His Asn
385 390 395 400

Ile Ile Arg Asn
404

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1212 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Alcaligenes faecalis
(B) STRAIN: TE3581 (FERM P-14237)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1 to 1212

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATG ACT GAC GAC ATG TTG CAC GTG ATG AAA TGG CAC AAC GGC GAG AAA 48
Met Thr Asp Asp Met Leu His Val Met Lys Trp His Asn Gly Glu Lys
1 5 10 15

GAT TAT TCG CCG TTT TCG GAT GCC GAG ATG ACC CGC CGC CAA AAC GAC 96
Asp Tyr Ser Pro Phe Ser Asp Ala Glu Met Thr Arg Arg Gln Asn Asp
20 25 30

GTT CGC GGC TGG ATG GCC AAG AAC AAT GTC GAT GCG GCG CTG TTC ACC 144
Val Arg Gly Trp Met Ala Lys Asn Asn Val Asp Ala Ala Leu Phe Thr
35 40 45

TCT TAT CAC TGC ATC AAC TAC TAT TCC GGC TGG CTG TAC TGC TAT TTC 192
Ser Tyr His Cys Ile Asn Tyr Tyr Ser Gly Trp Leu Tyr Cys Tyr Phe
50 55 60

GGA CGC AAG TAC GGC ATG GTC ATC GAC CAC AAC AAC GCC ACG ACG ATT 240
Gly Arg Lys Tyr Gly Met Val Ile Asp His Asn Asn Ala Thr Thr Ile
65 70 75 80

TCG GCC GGC ATC GAC GGC GGC CAG CCC TGG CGC CGC AGC TTC GGC GAC 288

-continued

Ser Ala Gly Ile Asp Gly Gly Gln Pro Trp Arg Arg Ser Phe Gly Asp
85 90 95

AAC ATC ACC TAC ACC GAC TGG CGC CGC GAC AAT TTC TAT CGC GCC GTG 336
Asn Ile Thr Tyr Thr Asp Trp Arg Arg Asp Asn Phe Tyr Arg Ala Val
100 105 110

CGC CAG CTG ACC ACG GGC GCC AAG CGC ATC GGC ATC GAG TTC GAC CAC 384
Arg Gln Leu Thr Thr Gly Ala Lys Arg Ile Gly Ile Glu Phe Asp His
115 120 125

GTC AAT CTC GAC TTC CGC CGC CAG CTC GAG GAA GCC CTA CCG GGC GTC 432
Val Asn Leu Asp Phe Arg Arg Gln Leu Glu Glu Ala Leu Pro Gly Val
130 135 140

GAC TTC GTC GAC ATC AGC CAG CCC TCG ATG TGG ATG CGC ACC ATC AAG 480
Glu Phe Val Asp Ile Ser Gln Pro Ser Met Trp Met Arg Thr Ile Lys
145 150 155 160

TCG CTC GAA GAG CAG AAG CTG ATC CGC GAA GGC GCC CGC GTG TGT GAC 528
Ser Leu Glu Glu Gln Lys Leu Ile Arg Glu Gly Ala Arg Val Cys Asp
165 170 175

GTC GGC GGC GCG GCC TGC GCG GCT GCC ATC AAG GCC GGC GTG CCC GAG 576
Val Gly Gly Ala Ala Cys Ala Ala Ala Ile Lys Ala Gly Val Pro Glu
180 185 190

CAT GAA GTG GCG ATC GCC ACC ACC AAT GCG ATG ATC CGC GAG ATC GCC 624
His Glu Val Ala Ile Ala Thr Thr Asn Ala Met Ile Arg Glu Ile Ala
195 200 205

AAA TCG TTC CCC TTC GTG GAG CTG ATG GAC ACC TGG ACC TGG TTC CAG 672
Lys Ser Phe Pro Phe Val Glu Leu Met Asp Thr Trp Thr Trp Phe Gln
210 215 220

TCG GGC ATC AAC ACC GAC GGC GCG CAC AAT CCG GTC ACC AAC CGC ATC 720
Ser Gly Ile Asn Thr Asp Gly Ala His Asn Pro Val Thr Asn Arg Ile
225 230 235 240

GTG CAA TCC GGC GAC ATC CTT TCG CTC AAC ACC TTC CCG ATG ATC TTC 768
Val Gln Ser Gly Asp Ile Leu Ser Leu Asn Thr Phe Pro Met Ile Phe
245 250 255

GGC TAC TAC ACC GCG CTG GAG CGC ACG CTG TTC TGC GAC CAT GTC GAT 816
Gly Tyr Tyr Thr Ala Leu Glu Arg Thr Leu Phe Cys Asp His Val Asp
260 265 270

GAC GCC AGC CTC GAC ATC TGG GAG AAG AAC GTG GCC GTG CAT CGC CGC 864
Asp Ala Ser Leu Asp Ile Trp Glu Lys Asn Val Ala Val His Arg Arg
275 280 285

GGG CTC GAG CTG ATC AAG CCG GGC GCG CGC TGC AAG GAC ATC GCC ATC 912
Gly Leu Glu Leu Ile Lys Pro Gly Ala Arg Cys Lys Asp Ile Ala Ile
290 295 300

GAG CTC AAC GAG ATG TAC CGC GAG TGG GAC CTG CTG AAG TAC CGC TCC 960
Glu Leu Asn Glu Met Tyr Arg Glu Trp Asp Leu Leu Lys Tyr Arg Ser
305 310 315 320

TTC GGC TAT GGC CAC TCC TTC GGC GTG CTG TGC CAC TAC TAC GGT CGC 1008
Phe Gly Tyr Gly His Ser Phe Gly Val Leu Cys His Tyr Tyr Gly Arg
325 330 335

GAG GCC GGC GTG GAG CTG CGC GAG GAC ATC GAC ACC GAG CTG AAG CCC 1056
Glu Ala Gly Val Glu Leu Arg Glu Asp Ile Asp Thr Glu Leu Lys Pro
340 345 350

GGC ATG GTG GTC TCC ATG GAG CCG ATG GTG ATG CTG CCG GAG GGC ATG 1104
Gly Met Val Val Ser Met Glu Pro Met Val Met Leu Pro Glu Gly Met
355 360 365

CCC GGT GCC GGC GGC TAT CGC GAG CAC GAC ATC CTG ATC GTC GGG GAG 1152
Pro Gly Ala Gly Gly Tyr Arg Glu His Asp Ile Leu Ile Val Gly Glu
370 375 380

GAC GGT GCC GAG AAC ATC ACC GGC TTC CCG TTC GGT CCG GAA CAC AAC 1200
Asp Gly Ala Glu Asn Ile Thr Gly Phe Pro Phe Gly Pro Glu His Asn
385 390 395 400